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Report

Telomerase Activation after Recruitment in Fission Yeast

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Summary

Current models depict that telomerase recruitment equates to activation. Telomeric DNA-binding proteins and the telomerase accessory proteins coordinate the recruitment of telomerase to the ends of chromosomes in a telomere length- and cell-cycle-dependent manner [1–4]. Recent studies have demonstrated that the telomeric protein TPP1 and its binding protein TIN2 are key proteins for both telomerase recruitment and processivity in mammalian cells [5–7]. Although the precise molecular mechanism of telomerase recruitment has not yet been established, targeted point mutations within the oligonucleotide/oligosaccharide-binding (OB)-fold domain of TPP1 have been shown to impair telomerase association and processivity [8–10]. In fission yeast, telomerase is recruited through an interaction between the telomerase subunit Est1 and Ccq1, a component of the Pot1-Tpz1 telomere complex (POT1-TPP1 orthologs) [11–15]. Here, we demonstrate that association of telomerase with telomeres does not engage activity. We describe a mutation of Tpz1 that causes critical telomere shortening despite telomeric accumulation of the telomerase catalytic subunit, Trt1. Furthermore, Est1-directed telomerase association with Ccq1 is transient, and the Est1-Ccq1 interaction does not remain the bridge between telomeres and telomerase. Rather, direct interaction of Trt1 with Tpz1 is critical for telomere elongation. Moreover, Ccq1, which has been well characterized as a telomerase recruiter, is also required for the activation of telomere-associated telomerase. Our findings reveal a layer of telomerase regulation that controls activity after recruitment.

Results and Discussion

Temporal Association of Est1 with Ccq1 at Telomerase-Active Telomeres

The *S. pombe* telomerase complex contains a reverse-transcriptase catalytic subunit (Trt1), an RNA template (TER1), and a telomerase-binding protein (Est1) [16–19]. The activity of telomerase at telomeres is positively and negatively controlled by the shelterin complex, which in *S. pombe* comprises Taz1, Rap1, Poz1, Tpz1, Pot1, and Ccq1 [11]. Telomerase is recruited to the telomere via an interaction between Est1 and Ccq1 [13, 15], which occurs as a result of primarily Rad3 (ATR)-mediated phosphorylation of Ccq1 at threonine 93 (T93), which provides a binding site for the 14-3-3-like phospho-binding domain of Est1 [13–15]. In the current *S. pombe* model, the Trt1-Est1 association is maintained through TER1 [18, 19]. Thus, association of telomerase with the telomere through Est1 requires TER1. However, the possibility exists that telomerase is retained at telomeres through another connection(s) because the association of telomerase with Ccq1 can be maintained without RNA [12, 13]. To investigate this further, the association between Trt1 and each of the proteins Ccq1, Tpz1, and Est1 was assessed by coimmunoprecipitation (coIP) in the presence of ribonuclease (RNase) (Figure 1A), which substantially reduced TER1 levels (Figure 1B). Interestingly, whereas the association between Trt1 and Est1 was reduced by RNase treatment, association of Trt1 with both Ccq1 and Tpz1 was resistant to RNase treatment, confirming that these interactions are not bridged by TER1. Given that Trt1 is not recruited to the telomere in *ter1*Δ and *est1*Δ mutants [13, 20], Trt1 must retain its connection with the Pot1 complex via an alternative interaction after recruitment through the Ccq1-Est1-TER1 pathway.

Intriguingly, the binding domains for Est1 and Tpz1 in Ccq1 seem to overlap [15]. Similarly, the 14-3-3-like domain of Est1 binds to both Ccq1 and TER1 [13]. Hence, their interactions could be mutually exclusive. To test this hypothesis, we conducted yeast three-hybrid analyses, in which expression of a third factor (protein or RNA) was induced by removal of methionine from the media. Although Ccq1 and Est1 were able to interact under methionine plus conditions, removal of methionine to allow coexpression of TER1 or Tpz1 disrupted the Ccq1-Est1 interaction (Figure 1C; Figures S1A and S1B available online). This indicates that Ccq1 cannot bind Est1 and Tpz1 simultaneously and also that Est1 cannot simultaneously bind both Ccq1 and TER1. These results raise the possibility that in order to form the Ccq1-Est1 complex, Ccq1 and Est1 must detach from Tpz1 and the telomerase complex, respectively. Collectively, our data suggest that the Ccq1-Est1 interaction does not bridge Tpz1-Ccq1 and Trt1 and predict an alternative association between shelterin and Trt1 after recruitment to the telomere by Est1.

Overexpression of Tpz1 Confers Telomere Elongation

Telomere length homeostasis is in part regulated by the availability of telomeric proteins and telomerase components. To investigate whether Est1 is involved in telomerase activation, we overexpressed Est1 as well as Trt1 and Tpz1. Contrary to the results of studies showing that hEst1A overexpression results in end-to-end chromosome fusions and shortening of telomeres in human cells [21, 22], overexpression of Est1 in *S. pombe* did not affect telomere length (Figures S1C and S1D). However, a number of Est1 (or SMG) family proteins exist in mammals, and their crucial roles in the nonsense-mediated mRNA decay pathway make it difficult to assess their function in telomerase recruitment. Intriguingly, however, we found that telomeres were elongated slightly by Trt1 overexpression and profoundly by Tpz1 overexpression (Figures S1C and S1D). These findings largely recaptured similar phenomena that have been observed previously in human cell lines [23, 24]. The telomere elongation caused by Tpz1 overexpression is not due to disruption of shelterin function because deletion of *poz1* in a strain overexpressing Tpz1 results in additive elongation of telomeres (Figure S1E). Rather, it is likely to stem either from promiscuous recruitment of telomerase to

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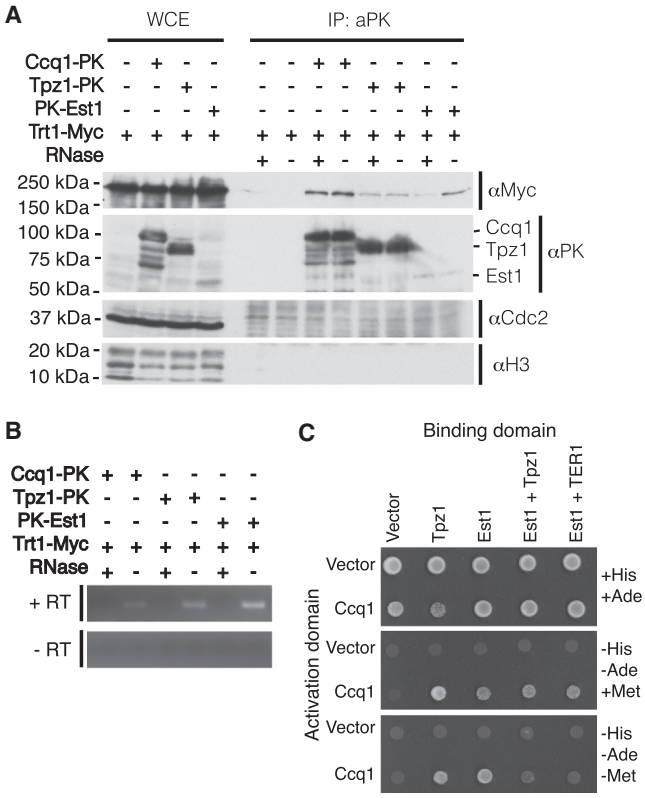

Figure 1. The Association of Est1 with Ccq1 during Telomerase Recruitment Is Likely to Be Transient

(A) Associations of Trt1 with Ccq1 and with Tpz1 are retained in the absence of TER1. Whole-cell extracts (WCEs) were immunoprecipitated (IP) with anti-PK (aPK) antibody in the presence or absence of RNase to purify Ccq1, Tpz1, or Est1 complexes. The resulting immunoprecipitates were hybridized with either anti-PK or anti-Myc. Cdc2 was used as a control for sample input. Histone H3 was used as a control for the presence of DNA.

(B) RNase treatment reduces TER1 levels. RNA extracted from 5% of each IP sample was subjected to RT-PCR. TER1 PCR products were visualized on a 2% agarose gel. As a control, the RT enzyme was substituted with water (−RT).

(C) Yeast three-hybrid analysis: coexpression of Tpz1 or TER1 disrupts Ccq1-Est1 binding. Equal amounts of cells were spotted on selection plates (−His −Ade) and a nonselective plate to control for loading (+His +Ade). Coexpression of Tpz1 or TER1 from the *MET17* promoter was induced by removal of methionine (Met) from the media. Expression of gene products is shown in Figure S1.

telomeres regardless of length, or enhanced telomerase activity. Notably, Tpz1 overexpression increased the length of the entire population of telomeres, rather than merely increasing telomere length heterogeneity (Figure S1C). Thus, our results favor the intriguing possibility that Tpz1, the ortholog of mammalian TPP1, may be involved in targeted telomerase recruitment/retention and activation in fission yeast.

Lysine 75 and Threonine 78 of Tpz1 Are Required for Telomere Extension

Because the structure and function of Tpz1 are conserved with that of human TPP1 (hTPP1) [11], it is tempting to speculate that a telomerase affinity region resides in the oligonucleotide/oligosaccharide-binding (OB) fold domain of fission yeast Tpz1 as it does in hTPP1. Several core residues within the loop region after the second α helix of the OB fold of hTPP1 have been identified as responsible for the association of TPP1 with telomerase [8–10]. This region has been coined the "TEL patch" (TPP1 glutamate [E]- and leucine [L]-rich patch) [10]. The corresponding residues were systematically mutated with COOH terminus HA tagging at one allele of *tpz1*$^{+}$ in diploid cells, and the stability of the mutant Tpz1 was determined (Figure S2A). The telomere lengths of diploid strains heterozygous for the *tpz1* mutation and the *tpz1* mutant haploid offspring were determined by Southern blot (Figures S2B and S2C). Alanine substitution of several amino acid residues within the corresponding region of Tpz1 (EKRI at position 74–77; and TS at position 78–79) was found to cause shortening of telomeres (Figure S2C). Further mutagenesis of individual residues within this region enabled us to identify lysine 75 (K75) and threonine 78 (T78) as the key residues required for telomere lengthening (Figures 2A and S2C). These residues appear to act in an epistatic manner because telomere shortening in the double-mutant *tpz1*-K75A-T78A was not additive. Furthermore, it seems that the charge of the residue is important for function because substitution of K75 with arginine did not affect telomere length, whereas substitution with alanine did (Figure 2A). Indeed, substitution of T78 with the negatively charged residues aspartic acid (T78D) or glutamic acid (T78E) resulted in defective telomere maintenance, equal to T78A. Conversely, *I77A* and *S79A* mutations resulted in slight elongation of telomeres (Figure S2C). Curiously, slow-migrating bands were observed prominently in extracts from strains expressing K75A, R76A, and T78A mutant forms of Tpz1. Phosphatase treatment suggested that the shifted bands are due to phosphorylation of the Tpz1 protein (data not shown). In summary, the TEL patch-like region in Tpz1 can modulate telomere length homeostasis, and the charge imparted by residues K75 and T78 is critical for elongation of telomeres.

To assess whether telomere shortening in the *tpz1-K75A* mutant is caused by impaired telomerase activity, heterozygous *tpz1*$^{K75A/+}$ diploids were sporulated, and telomere length was monitored in the *tpz1-K75A* offspring over time. A *trt1*$^{\Delta/+}$ diploid strain was also sporulated, and the *trt1*Δ haploid offspring were examined side by side with the *tpz1-K75A* haploid cells. Similar to *trt1*Δ cells, *tpz1-K75A* haploid mutants exhibited progressive shortening of telomeres with increasing generations (Figure 2B). However, the extent of telomere loss was smaller in *tpz1-K75A* mutants compared to *trt1*Δ cells. Unlike *trt1*Δ cells, the shortened telomeres were maintained during later generations rather than being lost completely. This phenotype appears similar to that of *ccq1*Δ cells in which short telomeres are maintained by homologous recombination [12]. However, in the case of *tpz1-K75A* cells, the short telomeres are maintained by telomerase because they were lost completely after further deletion of the *trt1*$^{+}$ gene (Figure 2C). Furthermore, the inability to recruit telomerase due to introduction of the *ccq1-T93A* mutation in the *tpz1-K75A* strain also resulted in telomere loss (Figure 2D). Thus, short telomeres in *tpz1-K75A* mutants are maintained by impaired telomerase activity.

Because Tpz1 is part of the shelterin complex and shelterin formation can negatively control telomerase activity, it is possible that telomere shortening in the *tpz1-K75A* mutant is occurring through enhanced suppression of telomerase activity. Our current knowledge of Tpz1 protein structure and function suggests that K75 falls within the Pot1-binding domain [11]. As such, mutation of this residue might enhance the ability of Tpz1 to interact with Pot1, resulting in a stronger shelterin formation that blocks telomerase activity. Using yeast two-hybrid analysis, we reassessed whether the OB-fold domain

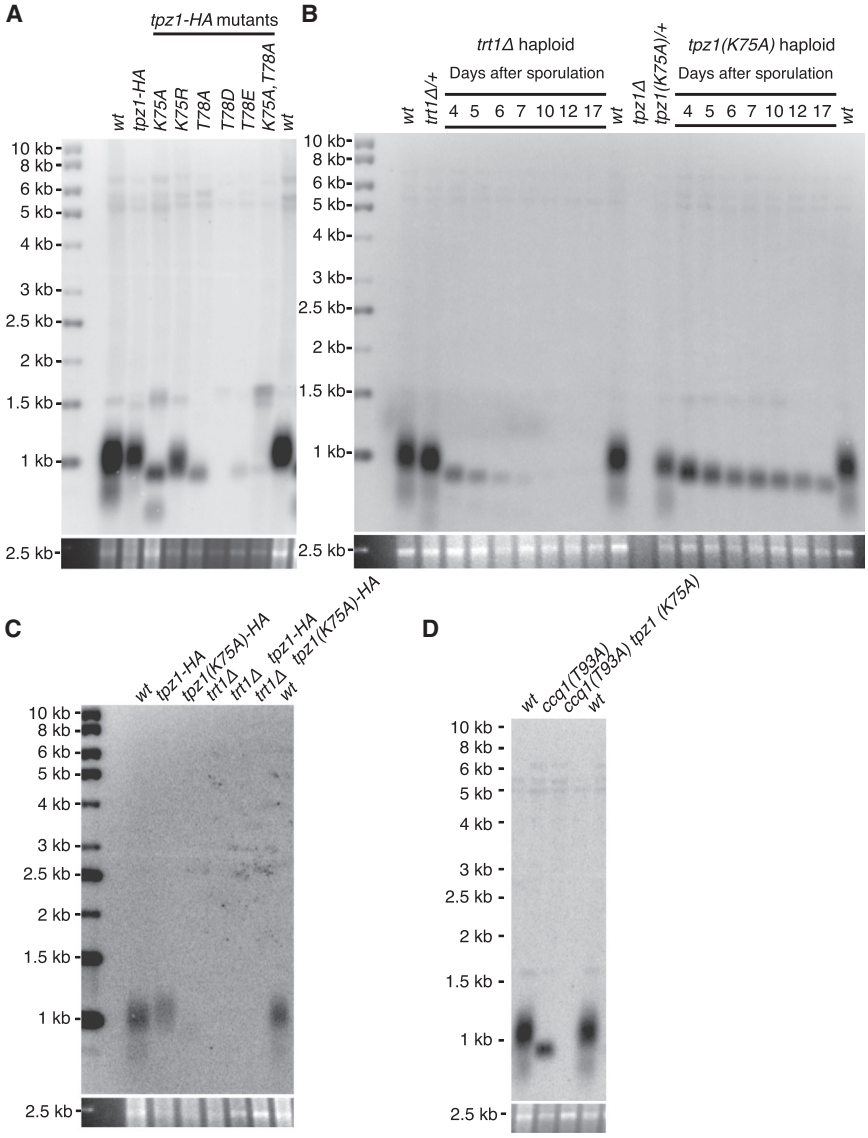

Figure 2. Mutation of the OB-Fold Domain of Tpz1 Results in Impaired Telomerase Activity

(A–D) Telomere Southern blots of genomic DNA digested with EcoRI and hybridized with a telomeric probe. A slice of the EtBr-stained gel image at 2.5 kb is shown below the blots as a loading control. (B) Genomic DNA was harvested at multiple intervals (as indicated) over the course of >2 weeks after sporulation of diploid strains.

of Tpz1 (residues 1–154) is required for binding to Pot1. By analyzing interactions with a range of Tpz1 truncation proteins, we were able to resolve the binding sites for Pot1, Ccq1, and Poz1 to residues 155–213, 422–490, and 479–508, respectively (Figure S3A). Thus, like mammalian TPP1, the OB-fold of *S. pombe* Tpz1 is not required for Pot1 binding or binding to Poz1 or Ccq1.

Mutation of *taz1*, *rap1*, and *poz1* leads to disruption of shelterin formation and massive telomerase-dependent elongation of telomeres [11, 20, 25, 26]. Mutation of *tpz1*$^{+}$ to *tpz1-K75A* in strains carrying deletions of the *taz1*, *rap1*, or *poz1* genes resulted in telomeres that were significantly longer than those in cells carrying only the *tpz1-K75A* mutation. However, the presence of the *tpz1-K75A* mutation reduced the telomere elongation seen in *taz1Δ*, *rap1Δ*, and *poz1Δ* single mutants (Figure S3B). This result suggests that telomerase activity is lower in the *K75A* mutant strain, even in the absence of shelterin formation. Together, our results show that the *tpz1-K75A* mutation does not affect the formation and function of the shelterin complex. Rather, it directly impairs telomerase activity.

Tpz1-K75 Controls Telomerase Activity after Recruitment

Because the OB-fold domain of hTPP1 can recruit human TERT [8], *S. pombe* Trt1 may associate with, and be retained at telomeres by, Tpz1 after recruitment via Est1. As such, the *K75A* mutation in Tpz1 might impair the stability of the interaction between the Pot1 complex and telomerase. To address this possibility, we examined the efficiency of association between Tpz1 and Trt1 by coIP. Surprisingly, Trt1 was enriched in immunoprecipitates containing Tpz1-K75A compared to wild-type (WT) Tpz1 (Figure 3A). Furthermore, chromatin immunoprecipitation (ChIP) experiments indicate that strains carrying mutant *tpz1-K75A* exhibit slightly greater enrichment of Trt1 at the telomere than strains carrying the WT *tpz1*$^{+}$ gene (Figure 3B). Interestingly, the efficiency of association observed by coIP between Ccq1 and Trt1 was also increased in *tpz1-K75A* mutants, whereas the interaction between Ccq1 and Tpz1 was not affected (Figure 3C). Thus, Trt1 is not only recruited but also appears to accumulate at the telomere in *tpz1-K75A* mutants.

Although Tpz1-K75A associates with Trt1, telomeres are short, implying that telomerase activity/processivity is compromised. Because TEL patch mutations in TPP1 impair the efficiency of both TERT association and telomerase processivity in human cells, it is possible that a direct interaction between Tpz1 and Trt1 within the shelterin-telomerase complex is necessary for full telomerase activity after recruitment in fission yeast. To genetically test this speculation, we generated strains expressing a Trt1-Tpz1 fusion protein. Tandem PK tags and the *tpz1* open reading frame were inserted before the stop codon of the endogenous *trt1* gene in a heterozygous *tpz1*$^{\Delta/+}$ diploid strain (Figure S4A). The resulting diploid cells exhibited WT telomere length, as did *tpz1*$^{+}$ haploid strains carrying the fusion protein (Figure 4A), and the fusion protein was stably expressed (Figure S4B). Dysfunction of either Trt1 or Tpz1 is known to lead to telomere loss [11, 16]. However, haploid *tpz1*Δ strains in which the endogenous *trt1*$^{+}$ gene was replaced with the *trt1-tpz1* chimera gene retained telomeres, indicating that the two fused proteins are functional. In fact, telomeres were slightly elongated in Trt1-Tpz1 fusion strains carrying *tpz1*Δ, which might reflect loss of selective targeting of telomerase to short telomeres (Figure 4A). Intriguingly, the telomere-shortening defect

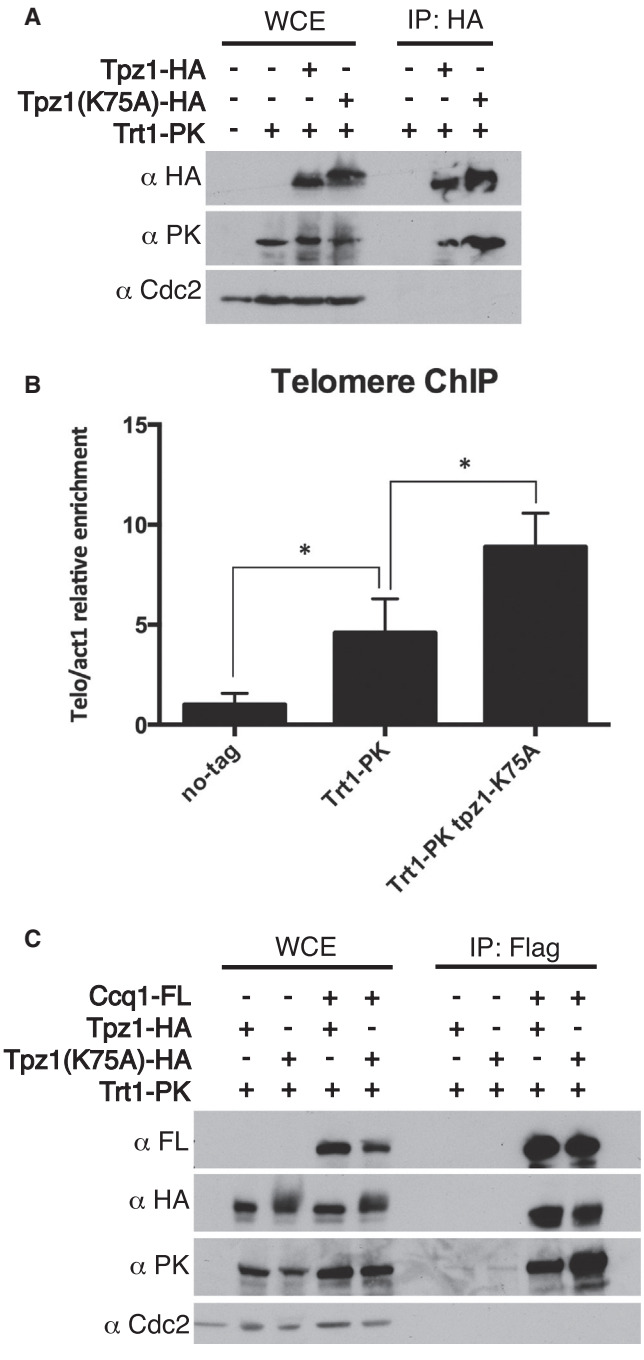

Figure 3. Tpz1 K75 Is Not Involved in Telomerase Recruitment

(A) Association efficiency of Trt1 with Tpz1 increases when K75 is mutated to alanine. WCEs were immunoprecipitated with anti-HA antibody to purify Tpz1. The resulting immunoprecipitates were hybridized with anti-PK. Cdc2 was used as a control for sample input.

(B) Telomere ChIP: Trt1 is present at the telomere in strains carrying the *tpz1-K75A* mutation. Strains were crosslinked, and WCEs were subjected to immunoprecipitation with anti-PK antibody. The *trt1-PK tpz1-K75A* cells were prepared soon after germination from the heterozygous diploid. DNA fragments in the immunoprecipitate were quantified using quantitative PCR (qPCR). Data were obtained from four independent experiments, and normalized to qPCR values were obtained from a control gene sequence (*act1*) and expressed as fold enrichment over the values obtained from crosslinked WT (untagged) cells; the average and SD of four independent experiments are shown. *p = 0.0289 for "no-tag versus Trt1-PK." *p = 0.0209 for "Trt1-PK versus Trt1-PK tpz1-K75A."

(C) Association efficiency of Trt1 with Ccq1 increases when K75 of Tpz1 is mutated to alanine. The interaction of Ccq1 with Tpz1 is not affected by substitution of Tpz1 K75 with alanine. WCEs were immunoprecipitated with anti-Flag antibody to purify Ccq1. The resulting immunoprecipitates were hybridized with either anti-HA or anti-PK. Cdc2 was used as a control for sample input.

observed in *tpz1-K75A* strains was rescued by fusing Tpz1-K75A with Trt1 (Figure 4B). Thus, fusion of Tpz1 with Trt1 can overcome the telomerase activity defect caused by the *tpz1-K75A* mutation. OB-folds are often composed of a number of hydrophobic and positively charged residues that are important for mediating interactions with DNA [27]. It is possible that charge is also important for interaction of the OB-fold with the Trt1 complex, and alteration of this charge, for example through the *K75A* mutation, could reduce telomerase activity. Taken together, these results highlight that a direct association between Tpz1 and Trt1 promotes telomere lengthening after telomerase has been recruited to telomeres.

Ccq1 Is Required for Telomerase Activation after Recruitment

Utilizing the Trt1-Tpz1 fusion system, we further asked whether Ccq1 is also required for telomerase activity after recruitment. First, to determine whether Est1 recruitment via Ccq1 is still required for telomerase function when Trt1 is fused to Tpz1, the *T93A* mutation was introduced into endogenous *ccq1*$^{+}$ in a *trt1-tpz1* haploid strain. These cells displayed telomere elongation comparable to strains expressing WT Ccq1 and the Trt1-Tpz1 fusion, indicating that fusion of Trt1 with Tpz1 bypasses the need for Ccq1 in telomerase recruitment (Figure 4C). Second, to investigate whether Ccq1 is required for telomerase activation, the *ccq1* gene was deleted in a haploid strain expressing the Trt1-Tpz1 fusion protein. Strikingly, telomeres were lost in *ccq1*Δ cells, indicating that Ccq1 is required for telomerase activity after interaction of Trt1 with Tpz1 (Figure 4C). Interestingly, whereas *ccq1*Δ single mutants activate homologous recombination before complete loss of the telomeric repeats [12], this was not observed in the presence of the Trt1-Tpz1 fusion, suggesting that telomerase competes with the telomere-recombination pathway in the absence of Ccq1. To investigate this further, we asked whether it was necessary for Tpz1 within the fusion construct to interact with Ccq1. A recent study described a single amino acid mutation (L449A) within Tpz1 that disrupts the interaction between Tpz1 and Ccq1 yet retains Ccq1 at telomeres [25]. Curiously, cells expressing the Trt1-Tpz1(L499A) fusion protein did not lose telomeres, although they were maintained at a shorter length than in cells expressing the WT Trt1-Tpz1 fusion (Figure 4C). Collectively, these results indicate that whereas fusion of Trt1 with Tpz1 bypasses the need for Ccq1 in the recruitment process, Ccq1 is in fact required for telomerase activity after recruitment.

Conclusions and Perspectives

Using protein fusions and loss-of-function mutations, we have been able to dissect the events of telomerase recruitment and activity. The reduced telomerase activity observed in strains containing the *tpz1-K75A* mutation despite accumulation of Trt1 at the telomere demonstrates that association of telomerase with telomeres is not sufficient to regulate telomere length. Furthermore, the interaction between Ccq1 and Est1 that is required for telomerase recruitment appears to be transient, with a more stable association between Trt1 and

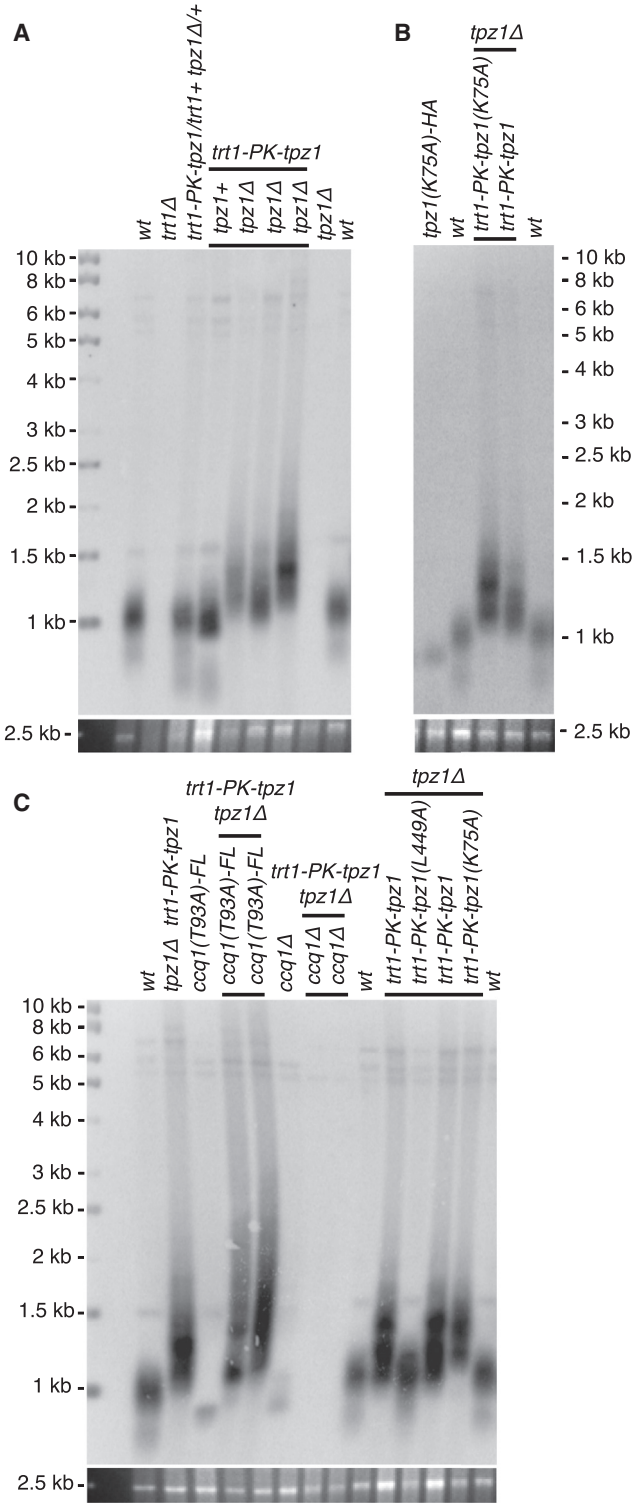

Figure 4. Direct Interaction of Tpz1 with Trt1 Rescues Telomerase Activity in *tpz1-K75A* Mutants

(A–C) Telomere Southern blots of genomic DNA digested with EcoRI and hybridized with a telomeric probe. A slice of the EtBr-stained gel image at 2.5 kb is shown below the blots as a loading control.

(A) Fused Tpz1 and Trt1 are functional: the fusion can maintain telomeres in the absence of endogenous Tpz1 or Trt1.

(B) Fusion of Trt1 with Tpz1 rescues the *tpz1-K75A* mutant phenotype.

(C) Fusion of Trt1 to Tpz1 bypasses the need for Ccq1 in telomerase recruitment, but Ccq1 is still required for telomerase activity.

Tpz1-Ccq1 being achieved after recruitment. Specifically, interaction of Trt1 with Tpz1 is crucial for telomere extension, and Ccq1 is essential for telomerase activation. Collectively, our data illustrate that telomerase recruitment and activation are separate events (discussed further in Figure S4C) and highlight the previously uncharacterized importance of Tpz1 and Ccq1 in regulating the latter. Such a two-step mechanism may well be conserved from yeast to human.

In many human cancer cells, telomerase is highly expressed and recruited to all telomeres, but processivity is low, resulting in maintenance of short telomeres [28, 29]. Our findings shed light on the existence of a telomerase regulatory step after recruitment. Further investigation of telomerase activation mechanisms would benefit our understanding of how cancer cells maintain short telomeres that lead to chromosome instability.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.07.035.

Author Contributions

K.T. and C.A.A. were responsible for study design, experimental work, data analysis, and manuscript preparation. S.R.P. carried out yeast two- and three-hybrid assays. H.A. and V.M. contributed to immunoprecipitation assays and manuscript preparation.

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